Association study of the calcitonin gene-related polypeptide-alpha (*CALCA*) and the receptor activity modifying 1 (*RAMP1*) genes with migraine.

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Abstract

Migraine is a common neurovascular brain disorder characterised by recurrent attacks of severe headache that may be accompanied by various neurological symptoms. Migraine is thought to result from activation of the trigeminovascular system followed by vasodilation of pain-producing intracranial blood vessels and activation of second-order sensory neurons in the trigeminal nucleus caudalis. Calcitonin gene-related peptide (CGRP) is a mediator of neurogenic inflammation and the most powerful vasodilating neuropeptide, and has been implicated in migraine pathophysiology. Consequently, genes involved in CGRP synthesis or CGRP receptor genes may play a role in migraine and/or increase susceptibility. This study investigates whether variants in the gene that encodes CGRP, calcitonin-related polypeptide alpha (*CALCA*) or in the gene that encodes a component of its receptor, receptor activity modifying protein 1 (*RAMP1*), are associated with migraine pathogenesis and susceptibility. The single nucleotide polymorphisms (SNPs) rs3781719 and rs145837941 in the *CALCA* gene, and rs3754701 and rs7590387 at the *RAMP1* locus, were analysed in an Australian Caucasian population of migraineurs and matched controls. Although we find no significant association of any of the SNPs tested with migraine overall, we detected a nominally significant association (p=0.031) of the *RAMP1* rs3754701 variant in male migraine subjects, although this is non-significant after Bonferroni correction for multiple testing.

Keywords: *CALCA*, *RAMP1*, CGRP, migraine, polymorphism, association study

1. Introduction

Migraine is a common episodic neurological disorder characterized by severe head pain, usually accompanied by nausea, vomiting, neurological disturbance, photophobia and phonophobia. Migraine affects approximately 6% of men and 17% of women and has significant personal, social and economic burdens (Lipton et al., 2007). Two main types of migraine have been classified by the International Headache Society (2004), distinguished by the presence of an aura preceding headache in the early stages of the attack: migraine with aura (MA) or without aura (MO) (Society, 2004). The pathophysiology of migraine is only partially understood, but is believed to be caused by activation of the trigeminovascular system which results in vasodilation of pain-producing intracranial blood vessels and activation of second-order sensory neurons in the trigeminal nucleus caudalis with subsequent perception of pain (Olesen, 2006). Cortical spreading depression (CSD), a slowly propagating wave of neuronal and glial depolarisation, is thought to underlie the aura and can activate trigeminal nociceptors (Bolay et al., 2002; Zhang et al., 2010).

Family and twin studies have shown that both the rare and common forms of migraine have a significant genetic basis. Some insights into the genetics of migraine have come from the investigation of the rare severe monogenic migraine subtype Familial Hemiplegic Migraine (FHM) in which the genes causal for this type of MA (*CACNA1A*, *ATPA2* and *SCNA1*) encode proteins involved in ion or neurotransmitter transport in the brain (reviewed in (de Vries et al., 2009)). Other loci associated with migraine have been found via family (*KCNK18*, (Lafreniere et al., 2010), genome-wide association (*MTDH*, (Anttila et al., 2010) and linkage studies (reviewed in (Maher and Griffiths, 2011)). Another approach to identifying genes implicated in migraine has been by investigation of candidate genes involved in the key biological pathways implicated in migraine pathology. For example, SNPs in genes which play a role in neurotransmitter levels in the brain including *DBH* (Fernandez et al., 2006), *SLC6A3* (Todt et al., 2009) and various serotonin-related genes (Corominas et al., 2010) have shown positive associations with migraine, as have other neurological, hormonal and vascular candidate genes, in certain migraine populations (de Vries et al., 2009; Maher and Griffiths, 2011). Paragraphs 2 and 3 condensed and some text left out.

Calcitonin gene-related polypeptide-alpha (α-CGRP) encoded by the *CALCA* gene is a 37 amino acid neuropeptide that is a potent vasodilator and is one of the mediators of neurogenic inflammation. CGRP-related genes are good candidates to screen for an association with migraine susceptibility as several lines of evidence suggest that α-CGRP is a key player in migraine pathology. Plasma levels of CGRP have been found to be elevated during spontaneous and nitroglycerin-induced migraine attacks (Goadsby et al., 1990; Juhasz et al., 2003) and reduced by the anti-migraine agent sumatriptan, co-incident with pain relief (Goadsby and Edvinsson, 1993). Some recent well-controlled studies have questioned this, but the controversy may be explained by reports of higher plasma CGRP levels outside of a migraine attack in migraineurs compared with control individuals. Importantly, individuals with migraine (Lassen et al., 2002), but not healthy controls develop a migraine-like headache in response to intravenous administration of CGRP, suggesting that migraineurs are especially sensitive to CGRP. Finally CGRP receptor antagonists are effective at alleviating the pain and associated symptoms of a migraine attack (Edvinsson and Ho, 2010).

Together these findings suggest that the direct effect of CGRP on its receptor is crucial in the initiation and perpetuation of a migraine attack. Paragraphs 4 and 5 condensed and some text left out.

α-CGRP is expressed in trigeminal ganglia neurons (TGNs) and is the main mediator of dilation in human cerebral arteries. It acts via CGRP receptors which are located at several sites, including the cerebrovasculature, the trigeminocervical complex within the brainstem and the trigeminal ganglion (Oliver et al., 2002; Storer et al., 2004; Petersen et al., 2005; Zhang et al., 2007). The CGRP receptor is a G protein coupled receptor that is a multimer of calcitonin receptor-like receptor (CLR), a receptor component protein (RCP) which is involved in coupling the receptor to downstream signalling pathways, and receptor activity-modifying protein 1 (RAMP1). RAMP1 is a small single-transmembrane protein that is required for CGRP binding by CLR and, in addition to ligand-specificity, influences CLR glycosylation and cell surface trafficking (McLatchie et al., 1998; Steiner et al., 2002). RAMP1 overexpressing mice display photophobia and mechanical allodynia, two of the symptoms of migraine, suggesting that regulation of this single gene could potentially contribute to migraine susceptibility (Russo et al., 2009). In the present study we have selected two SNPs in the *CALCA* gene, as well as two SNPs around the *RAMP1* gene, to investigate whether they contribute to the risk of migraine in an Australian Caucasian population. Paragraphs 6 and 7 condensed and some text left out.

2. Materials and Methods

2.1 Subjects

A cohort of 284 migraineurs and 284 controls matched for sex, age (+/-5 years) and ethnicity, recruited from in and around South Eastern Australia was used for this study (Colson et al., 2004). All individuals provided informed consent and were adult Caucasians of European decent living in Australia, having ancestors who emigrated within the last 160 years from various locations within the British Isles and other parts of Europe. Migraineurs were diagnosed by a clinical neurologist as having either MA or MO based strictly on the widely accepted criteria specified by the IHS (Society, 2004). The study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects. A whole blood sample was collected from each participant and genomic DNA was extracted from white blood cells using a standard salting out method (Miller et al., 1988). Samples used for the genotyping study were all from unrelated individuals and the control group consisted of individuals with no family history of migraine.

2.2 Genotyping

Genotype for each SNP was determined by restriction-fragment length polymorphism (RFLP) analysis of restriction enzyme-digested PCR products on agarose gels. A 20-μl PCR reaction mix contained 1x PCR buffer, 1.75 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM of each primer, 20-40 ng of genomic DNA and 1.5U of GoTaq® (Promega). Thermocycler conditions were an initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45s, 72°C for 45 s and a final extension step of 72°C for 7 min. Specific primers were used to amplify each region containing the SNPs and restriction enzymes (NEB) chosen that would differentiate the two alleles after separation on 3% agarose gels. Two SNPs from the *CALCA* gene and two SNPs from the *RAMP1* gene were analysed as follows:

rs3781719 is the first SNP of a closely linked double base exchange at position -624 (T/C) of the *CALCA* gene promoter. The primers 5’-GCTGTTTCTCACAATATTCC and 5’-CAATTCCTGGTTGTGTGATC generate a 109-bp PCR product which with *Bsm*AI is digested into 86- and 23-bp fragments in the case of the ‘C’ allele mutation.

rs145837941 is a SNP located in the coding sequence of the *CALCA* gene common to both calcitonin and CGRP-α. A 4218T>C base-exchange results in a non-synonymous amino acid change (Leu66Pro). The primers 5’-AGCCTGCACTGAGTTTGCTTCCC and 5’- ATCCACCTTCCTGTGTATGCTGCG generate a 236-bp PCR product which with *Alu*I is digested into 140- and 96-bp fragments in the case of the wild type ‘T’ allele.

rs3754701 is a SNP in the *RAMP1* gene promoter at position -1166 (T/A). The primers 5’-TGGCCTCTCGGCATTACTG and 5’-TGCACAGGTGGTAGGCATG generate a 373-bp PCR product which with *Acl*I is digested into 215-bp and 158-bp fragments in the case of the ‘A’ allele mutation.

rs7590387 is a SNP (G/C) located 1.4 kb downstream of the *RAMP1* gene. The primers 5’-AGAGCCTGTCGTTGTGCCCA and 5’-CTCCCGTCTCCTCGCCCTCA generate a 284-bp product which with *Xcm*I is digested into 194- and 90-bp fragments in the case of the ‘C’ allele mutation.

2.3 Statistical analysis

Hardy-Weinberg equilibrium was verified for observed genotype frequencies for each SNP to detect deviation from the normal genotype distribution in the population. Chi-square (χ^2) analysis was performed for each SNP to test for significant differences in genotype and allele frequencies in migraineurs MA, MO and combined migraine groups versus control results to detect any association with migraine. The results were also broken down into female and male sub-populations. The Statistical Package for Social Sciences (SPSS version 19.0) was used for all computations. Power analysis indicated that if the polymorphism were to confer a two-fold increase in risk of migraine, the case and control groups used in this study were of sufficient size to have ~80% power to detect an allelic association at the 0.05 level.

3. Results

In a study to investigate the role of the *CALCA* gene in psychiatric or neurological disease based on the neuromodulatory role of α-CGRP in dopaminergic transmission, Buervenich et al., (2001) identified four novel polymorphisms at the *CALCA* locus (Buervenich et al., 2001). While no positive association was found for any of these polymorphisms with Parkinson’s disease or schizophrenia, because of the important role of CGRP in migraine pathogenesis, we wished to investigate them with respect to migraine susceptibility. We previously found no association with migraine for a 16-bp deletion polymorphism in intron 1 of *CALCA* (Menon et al., 2011). However, in the present study we investigated a polymorphism in the promoter of *CALCA* (rs3781719) which may influence transcription of α-CGRP, and the 4218T>C base-exchange (rs145837941) which results in a non-synonymous change (Leu66Pro) of a highly conserved amino acid which likely to disrupt structure of the CALCA propeptide. The *RAMP1* SNPs that we tested in this study were previously identified as part of a susceptibility haplotype for cerebral infarction (Nakazato et al., 2010). We were interested in the *RAMP1* promoter SNP rs3754701, as it sits within a DNaseI hypersensitive site in a number of cell lines according to the ENCODE project and is also under the binding regions for a number of transcription factors including CTCF, c-myc, Znf143, GABP, CHD2 and Rad2 according to ENCODE

ChIP-seq data. We also investigated rs7590387 which is downstream of *RAMP1*, but in a region that we found to show some association with migraine (unpublished results) in a GWAS study of the Norfolk Island population (Cox et al., 2012). New text to address reviewers comments about SNP selection.

The genotypes for the two *CALCA* gene SNPs and the two *RAMP1* gene SNPs were determined using RFLP analysis. Chi-square (χ^2) analysis demonstrated that genotypes for all four SNPs were in Hardy-Weinberg equilibrium in both control and migraine groups.

***CALCA* rs3781719**

A total of 434 individuals were successfully genotyped for the *CALCA* promoter SNP, rs3781719, including 203 controls and 228 migraine cases, of which 147 were diagnosed with MA and 81 with MO. It should be noted that although MA typically accounts for only a third of migraineurs, our migraine population has a higher proportion of MA cases compared to MO, probably as the more severe MA-sufferers are more likely to identify as migrainuers and/or volunteer. χ^2-analysis was performed to compare the genotype and allele frequencies in the migraine population to controls, as well as the subgroups of migraineurs with aura to the controls and migraineurs without aura to controls (Table 1). No significant difference in genotype frequencies were found between cases and controls (p=0.260), MA and controls (p=0.563), nor MO and controls (p=0.133). There was also no significant difference in allele frequencies between cases and controls (p=0.333), MA and controls (p=0.370), nor MO and controls (p=0.593). The genotype and allele frequencies were also compared between migraineurs and controls sub-grouped into each of the genders (Table 1). No significant differences were seen between genotype or allele frequencies for female migraineurs compared to controls (p=0.270 and p=0.993, respectively). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.163) or alleles (p=0.055), although the latter is close to significance. Table 1a and 1b now condensed into a single table.

Table 1. Genotype and allele frequency distributions for the *CALCA* rs3781719 SNP in migraine and control groups and subgrouped by gender.

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		TT (%)	TC (%)	CC (%)			T (%)	C (%)	
Migraine	228	109 (47.8)	102 (44.7)	17 (7.5)	0.260	456	320 (70.2)	136 (29.8)	0.333
MA	147	72 (49.0)	62 (42.2)	13 (8.8)	0.563	294	206 (70.0)	88 (30.0)	0.370
MO	81	37 (45.7)	40 (49.4)	4 (4.9)	0.133	162	114 (70.4)	48 (29.6)	0.593
Control	203	111 (54.7)	75 (36.9)	17 (8.4)	-	406	297 (73.2)	109 (26.8)	-
Females									
Migraine	172	82 (47.7)	79 (45.9)	11 (6.4)	0.270	344	243 (70.6)	101 (29.4)	0.993
Control	148	76 (51.4)	57 (38.5)	15 (10.1)	-	296	209 (70.6)	87 (29.4)	-

Males									
Migraine	56	27 (48.2)	23 (41.1)	6 (10.7)	0.163	112	77 (68.8)	35 (31.2)	0.055
Control	55	35 (63.6)	18 (32.7)	2 (3.6)	-	110	88 (80.0)	22 (20.0)	-

MA- Migraine with aura, MO- Migraine without aura

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

N is total number of genotypes or alleles.

***CALCA* rs145837941**

rs145837941 is a SNP located in the coding sequence of the *CALCA* gene common to both calcitonin and α-CGRP. A 4218T>C base-exchange results in a non-synonymous amino acid change (Leu66Pro). A total of 462 individuals were genotyped for rs145837941, which included 236 migraine cases and 226 controls (Table 2). Because the CC genotype was very rare, the TC and CC genotypes were pooled together for analysis versus the wild type TT genotype for chi-square analysis between migraineurs and controls, however no significant difference was observed (p=0.913). Similarly there was no significant difference for alleles between cases and controls (p=0.926). No further analysis in subgroups was done because of the rarity of the C-allele. Furthermore, neither marker is in LD with 16 bp deletion polymorphism that we previously genotyped in this population (r^2=0.088, p=0.205 for rs3781719 and r^2=0.042, p=0.541 for rs145837941). The HapMap data release 27 shows poor variant coverage in this region.

Table 2. Genotype and allele frequency distributions for the *CALCA* rs145837941 SNP in migraine and control groups

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		TT (%)	TC (%)	CC (%)			T (%)	C (%)	
Migraine	236	224 (94.9)	11 (4.7)	1 (0.4)	0.913	472	459 (97.2)	13 (2.8)	0.926
Control	226	214 (94.7)	12 (5.3)	0 (0)	-	452	440 (97.3)	12 (2.7)	-

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

Because of the rarity of the C-allele, the P-value for genotypes was calculated for the TT genotype versus TC and CC genotypes pooled together.

***RAMP1* rs3754701**

rs3754701 is a SNP in the *RAMP1* gene promoter at position -1166 (T/A). A total of 485 individuals were successfully genotyped for rs3754701 comprised of 243 migraine cases (broken down into 145 MA and 98 MO patients), and 242 controls (Table 3). No significant difference in genotypic frequencies were found between cases and controls (p=0.360), MA and controls (p=0.276), nor MO and controls (p=0.260). No significant difference was found for genotypes frequencies between cases and controls (p=0.370), MA and controls (p=0.276), nor MO and controls (p=0.260) and for allelic frequencies between cases and controls (p=0.376), MA and controls (p=0.912), nor MO and controls (p=0.129). The genotype and allele frequencies were also compared between migraineurs and controls of the two genders (Table 3). No significant differences were seen between genotypic or allelic frequencies for female migraineurs compared to controls (p=0.560 and p=0.819, respectively). For the male sub-population, although close, the difference between migraineurs and controls was not significant for genotypes (p=0.068), but a significant difference in the allelic frequency was observed between migraineurs and controls (p=0.031). However, with Bonferroni correction of the p-value to an alpha of 0.0056 (considering the entire group and sub-group analyses) the result is non-significant.

Table 3a and 3b now condensed into a single table.

Table 3. Genotype and allele frequency distributions for the *RAMP1* rs3754701 SNP in migraine and control groups and subgrouped by gender.

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		TT (%)	TA (%)	AA (%)			T (%)	A (%)	
Migraine	243	91 (37.4)	120 (49.4)	32 (13.2)	0.360	494	302 (61.1)	184 (38.9)	0.376
MA	145	61 (42.1)	65 (44.8)	19 (13.1)	0.276	290	187 (64.5)	103 (35.5)	0.912
MO	98	30 (30.6)	55 (56.1)	13 (13.3)	0.260	196	115 (58.7)	81 (41.3)	0.129
Control	242	94 (38.8)	126 (52.1)	22 (9.1)	-	484	314 (64.9)	170 (35.1)	
Females									
Migraine	183	71 (38.8)	89 (48.6)	23 (12.6)	0.560	366	231 (63.1)	135 (36.9)	0.819
Control	179	63 (35.2)	97 (54.2)	19 (10.6)	-	358	223 (62.3)	135 (37.7)	-
Males									
Migraine	60	20 (33.3)	31 (51.7)	9 (15.0)	0.068	120	71 (59.2)	49 (40.8)	0.031
Control	63	31 (49.2)	29 (46.0)	3 (4.8)	-	126	91 (72.2)	35 (27.8)	-

MA- Migraine with aura, MO- Migraine without aura

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

N is total number of genotypes or alleles.

RAMP1 rs7590387

rs7590387 is a SNP (C/G) located 1.4 kb downstream of the *RAMP1* gene. A total of 456 individuals were successfully genotyped for rs7590387, consisting of 226 migraine cases (138 MA and 88 MO patients), and 230 controls. A chi-square analysis (χ^2) was performed to compare the allele and genotype frequencies of the migraine population to the controls (Table 4). No significant differences in genotypic frequencies were found between migraineurs and controls (p=0.341), MA and controls (p=0.566), nor MO and controls (p=0.299) and for allelic frequencies between migraineurs and controls (p=0.461), MA and controls (p=0.864), nor MO and controls (p=0.236). When migraineurs and controls were split into the two genders for analysis (Table 4), there were no significant differences between genotypic or allelic frequencies for female migraineurs compared to controls (p=0.865 and p=0.769, respectively), or for male migraineurs compared to controls (p=0.188 and p=0.366, respectively). According to CEPH Caucasian HapMap LD data rs3754701 and rs7590387 are not correlated (r^2=0.005) and we also find in our population that these two SNPs are not in LD (r^2=0.094, p=0.126).

Table 4a and 4b now condensed into a single table.

Table 4. Genotype and allele frequency distributions for the *RAMP1* rs7590387 SNP in migraine and control groups and subgrouped by gender.

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		GG (%)	GC (%)	CC (%)			G (%)	C (%)	
Migraine	226	58 (25.7)	123 (54.4)	45 (19.9)	0.341	452	239 (52.1)	213 (47.1)	0.461
MA	138	33 (23.9)	75 (54.3)	30 (21.7)	0.566	276	141 (51.1)	135 (48.9)	0.864
MO	88	25 (28.4)	48 (54.5)	15 (17.0)	0.299	176	98 (55.7)	78 (44.3)	0.236
Control	230	60 (26.1)	112 (48.7)	58 (25.2)	-	460	232 (50.4)	228 (49.6)	-
Females									
Migraine	171	44 (25.7)	93 (54.4)	34 (19.9)	0.865	342	181 (52.9)	161 (47.1)	0.769
Control	172	43 (25.7)	87 (52.1)	37 (22.2)	-	334	173 (51.8)	161 (48.2)	-
Males									
Migraine	55	14 (25.5)	30 (54.5)	11 (20.0)	0.188	110	58 (52.7)	52 (47.3)	0.366
Control	63	17 (27.0)	25 (39.7)	21 (33.3)	-	126	59 (46.8)	67 (53.2)	-

MA- Migraine with aura, MO- Migraine without aura

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

N is total number of genotypes or alleles.

4. Discussion

The identification of susceptibility genes for complex traits such as migraine can be challenging, in particular due to the contribution of multiple, and potentially interacting, loci as well as the confounding influence of environmental factors. However, unravelling the genetic basis of migraine should improve our understanding of the pathogenesis of the disease and may suggest pathways to which therapies can be targeted. Both the *CALCA* and *RAMP1* genes are good candidates to investigate whether polymorphisms at these loci play a role in migraine pathogenesis and/or susceptibility due to the well-documented relationship of CGRP with migraine. However, neither the 16-bp deletion in intron 1 of the *CALCA* gene that we tested previously (Menon et al., 2011), nor the two other *CALCA* SNPs and two *RAMP1* SNPs that we tested in this study, show a significant difference between the migraine and control groups, the MA and control groups, or the MO and control groups in this Australian population. In line with this, no positive association of the four SNPs in this study was found when the analysis was performed with female samples. An increase in frequency of the minor C-allele of the rs3781719 SNP in the promoter of the *CALCA* gene in male migraineurs was close to significance (p=0.055) and for one of the SNPs, rs3754701 which is in the promoter of the *RAMP1* gene, we observed a nominally significant increase in the frequency of the minor A-allele in male migraineurs (p= 0.031). However, the result is not significant with Bonferroni correction for multiple testing which gives a corrected p-value threshold of 0.0056. Because fewer males are affected by migraine than females, the number of male subjects in our population is low, so the association may be a false positive. However, sex-specific differences in mRNA levels of CGRP and CGRP receptor components including *RAMP1* have been found before and after induction of migraine-like symptoms in a rat preclinical migraine model (Stucky et al., 2011); furthermore, sex-specific differences are also apparent in the migraine-relevant behaviour of light aversion in mice overexpressing transgenic RAMP1 (Recober et al., 2009), suggesting that the result is potentially interesting. Future studies could look at these markers using a larger cohort, in particular of male subjects, in order to follow up any potential associations. While female sex hormones have a role in migraine and may help explain the preponderance of female migraine sufferers (Shyti et al., 2011), polymorphisms in some genes have been more strongly linked in males to either migraine, such as *DBH* (Fernandez et al., 2006) or particular migraine phenotypes, such as the C667T polymorphism in *MTHFR* (Liu et al., 2010).

The products of the *CALCA* gene including calcitonin, α-CGRP, their propeptides and other cleavage products exert many functions in the body. Mutations in a calcitonin receptor have been associated with altered bone density and decreased fracture risk in postmenopausal women (Masi et al., 1998; Taboulet et al., 1998). CGRP is vasodilator in peripheral organs as well as the brain and polymorphisms in *CALCA* gene have been linked to essential hypertension in Japanese and Chinese populations (Morita et al., 2007; Luo et al., 2008). A previous study had found no difference between migraine cases and controls for another *CALCA* gene promoter SNP (rs1553005) in a European population (Lemos et al., 2010). According to CEPH Caucasian HapMap LD data rs1553005 is in complete LD with rs3781719, however, we decided to test rs3781719 in our migraine population for a number of reasons. Firstly, we have a greater proportion of MA compared to MO cases, which would give us greater power to detect any association that is specific for this subgroup of migraine, and furthermore, our population is primarily made up of Northern European, i.e. British descendants, compared to the Southern European Portuguese cohort of that study and there is some genetic disparity between European populations. However, we found no significant association of either of the *CALCA* SNPs in this study with overall migraine or either MA or MO. The Lemos et al. (2010) study also investigated SNPs at the BDNF locus and, while no individual associations were detected, an increased risk for one of the genotype pairs of this particular *CALCA* SNP (rs1553005) and a *BDNF* SNP in migraineurs was found, suggesting interaction between these genes in migraine susceptibility. BDNF is co-expressed with CGRP in trigeminal ganglion neurons in rats and CGRP enhances the release of BDNF (Buldyrev et al., 2006) so future studies of CGRP-related genes in migraine could focus on interactions between loci.

Many of the anti-migraine drugs currently available target some action of CGRP action. Triptans act in part by inhibiting trigeminal release of CGRP (Bigal et al., 2009), while olcegepant (BIBN-4096BS) and telcagepant (MK-0974) are CGRP receptor antagonists (Olesen et al., 2004; Ho et al., 2008; Edvinsson and Ho, 2010). Some migraineurs respond better with certain migraine drugs than others and some fail to respond at all; studies have investigated SNPs in some factors and genes that may be involved (Ishii et al., 2012). Although we failed to detect association with migraine for the *CALCA* and *RAMP1* variations that we have looked at in this study, these genes could also potentially play a role in drug response to anti-migraine agents.

In conclusion, we investigated whether the SNPs rs3754701 and rs7590387 in *RAMP1* and rs3781719 and rs145837941 in the *CALCA* locus are associated with migraine susceptibility. We find no significant association of any of the SNPs tested with migraine overall. We did detect a nominally significant association of the minor A-allele for *RAMP1* rs3754701 in male migraine subjects, but this was no longer significant after Bonferroni correction. Although RAMP1, via its interaction with CGRP, has been implicated in migraine (Recober et al., 2009), our study is the first to investigate polymorphisms in the *RAMP1* gene in a migraine population. Future studies using a larger population to increase the power to detect significant associations, and greater gene coverage and haplotype analysis, would clarify whether polymorphisms in CGRP-related genes play a role in migraine.

Author's contributions

H.G.S., J.B. and S.M. performed the experimental procedures, participant sample preparation and contributed towards statistical analysis and manuscript finalisation. R.A.L. and L.M.H. contributed towards data interpretation and finalisation of the manuscript. L.R.G. and E.A.M. participated in the conception and design of the study. L.R.G. was also involved in data analysis and interpretation and coordinated the study.

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Association study of the calcitonin gene-related polypeptide-alpha (*CALCA*) and the receptor activity modifying 1 (*RAMP1*) genes with migraine.

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Abstract

Migraine is a common neurovascular brain disorder characterised by recurrent attacks of severe headache that may be accompanied by various neurological symptoms. Migraine is thought to result from activation of the trigeminovascular system followed by vasodilation of pain-producing intracranial blood vessels and activation of second-order sensory neurons in the trigeminal nucleus caudalis. Calcitonin gene-related peptide (CGRP) is a mediator of neurogenic inflammation and the most powerful vasodilating neuropeptide, and has been implicated in migraine pathophysiology. Consequently, genes involved in CGRP synthesis or CGRP receptor genes may play a role in migraine and/or increase susceptibility. This study investigates whether variants in the gene that encodes CGRP, calcitonin-related polypeptide alpha (*CALCA*) or in the gene that encodes a component of its receptor, receptor activity modifying protein 1 (*RAMP1*), are associated with migraine pathogenesis and susceptibility. The single nucleotide polymorphisms (SNPs) rs3781719 and rs145837941 in the *CALCA* gene, and rs3754701 and rs7590387 at the *RAMP1* locus, were analysed in an Australian Caucasian population of migraineurs and matched controls. Although we find no significant association of any of the SNPs tested with migraine overall, we detected a nominally significant association (p=0.031) of the *RAMP1* rs3754701 variant in male migraine subjects, although this is non-significant after Bonferroni correction for multiple testing.

Keywords: *CALCA*, *RAMP1*, CGRP, migraine, polymorphism, association study

1. Introduction

Migraine is a common episodic neurological disorder characterized by severe head pain, usually accompanied by nausea, vomiting, neurological disturbance, photophobia and phonophobia. Migraine affects approximately 6% of men and 17% of women and has significant personal, social and economic burdens (Lipton et al., 2007). Two main types of migraine have been classified by the International Headache Society, IHS (Society, 2004), distinguished by the presence of an aura preceding headache in the early stages of the attack: migraine with aura (MA) or without aura (MO) (Society, 2004). The pathophysiology of migraine is only partially understood, but is believed to be caused by activation of the trigeminovascular system which results in vasodilation of pain-producing intracranial blood vessels and activation of second-order sensory neurons in the trigeminal nucleus caudalis with subsequent perception of pain (Olesen, 2006). Cortical spreading depression (CSD), a slowly propagating wave of neuronal and glial depolarisation, is thought to underlie the aura and can activate trigeminal nociceptors (Bolay et al., 2002; Zhang et al., 2010).

Family and twin studies have shown that both the rare and common forms of migraine have a significant genetic basis. Some insights into the genetics of migraine have come from the investigation of the rare severe monogenic migraine subtype Familial Hemiplegic Migraine (FHM) in which the genes causal for this type of MA (*CACNA1A*, *ATPA2* and *SCNA1*) encode proteins involved in ion or neurotransmitter transport in the brain (reviewed in de Vries et al., 2009). Other loci associated with migraine have been found via family, e.g. *KCNK18* (Lafreniere et al., 2010), genome-wide association, e.g. *MTDH* (Anttila et al., 2010), and linkage studies (reviewed in Maher and Griffiths, 2011). Another approach to identifying genes implicated in migraine has been by investigation of candidate genes involved in the key biological pathways implicated in migraine pathology. For example, SNPs in genes which play a role in neurotransmitter levels in the brain including *DBH* (Fernandez et al., 2006), *SLC6A3* (Todt et al., 2009) and various serotonin-related genes (Corominas et al., 2010) have shown positive associations with migraine, as have other neurological, hormonal and vascular candidate genes, in certain migraine populations (de Vries et al., 2009; Maher and Griffiths, 2011).

Calcitonin gene-related polypeptide-alpha (α-CGRP) encoded by the *CALCA* gene is a 37 amino acid neuropeptide that is a potent vasodilator and is one of the mediators of neurogenic inflammation. CGRP-related genes are good candidates to screen for an association with migraine susceptibility as several lines of evidence suggest that α-CGRP is a key player in migraine pathology. Plasma levels of CGRP have been found to be elevated during spontaneous and nitroglycerin-induced migraine attacks (Goadsby et al., 1990; Juhasz et al., 2003) and are reduced by the anti-migraine agent sumatriptan, co-incident with pain relief (Goadsby and Edvinsson, 1993). Some recent well-controlled studies have questioned this, but the controversy may be explained by reports of higher plasma CGRP levels outside of a migraine attack in migraineurs compared with control individuals. Importantly, individuals with migraine (Lassen et al., 2002), but not healthy controls develop a migraine-like headache in response to intravenous administration of CGRP, suggesting that migraineurs are especially sensitive to CGRP. Finally CGRP receptor antagonists are effective at alleviating the pain and associated symptoms of a migraine attack (Edvinsson and Ho, 2010).

Together these findings suggest that the direct effect of CGRP on its receptor is crucial in the initiation and perpetuation of a migraine attack.

α-CGRP is expressed in trigeminal ganglia neurons (TGNs) and is the main mediator of dilation in human cerebral arteries. It acts via CGRP receptors which are located at several sites, including the cerebrovasculature, the trigeminocervical complex within the brainstem and the trigeminal ganglion (Oliver et al., 2002; Storer et al., 2004; Petersen et al., 2005; Zhang et al., 2007). The CGRP receptor is a G protein coupled receptor that is a multimer of calcitonin receptor-like receptor (CLR), a receptor component protein (RCP) which is involved in coupling the receptor to downstream signalling pathways, and receptor activity-modifying protein 1 (RAMP1). RAMP1 is a small single-transmembrane protein that is required for CGRP binding by CLR and, in addition to ligand-specificity, influences CLR glycosylation and cell surface trafficking (McLatchie et al., 1998; Steiner et al., 2002). RAMP1 overexpressing mice display photophobia and mechanical allodynia, two of the symptoms of migraine, suggesting that regulation of this single gene could potentially contribute to migraine susceptibility (Russo et al., 2009). In the present study we have selected two SNPs in the *CALCA* gene, as well as two SNPs around the *RAMP1* gene, to investigate whether they contribute to the risk of migraine in an Australian Caucasian population.

2. Materials and Methods

2.1 Subjects

A cohort of 284 migraineurs and 284 controls matched for sex, age (+/-5 years) and ethnicity, recruited from in and around South Eastern Australia was used for this study (Colson et al., 2004). All individuals provided informed consent and were adult Caucasians of European decent living in Australia, having ancestors who emigrated within the last 160 years from various locations within the British Isles and other parts of Europe. Migraineurs were diagnosed by a clinical neurologist as having either MA or MO based strictly on the widely accepted criteria specified by the IHS (Society, 2004). The study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects. A whole blood sample was collected from each participant and genomic DNA was extracted from white blood cells using a standard salting out method (Miller et al., 1988). Samples used for the genotyping study were all from unrelated individuals and the control group consisted of individuals with no family history of migraine.

2.2 Genotyping

Genotype for each SNP was determined by restriction-fragment length polymorphism (RFLP) analysis of restriction enzyme-digested PCR products on agarose gels. A 20-μl PCR reaction mix contained 1x PCR buffer, 1.75 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM of each primer, 20-40 ng of genomic DNA and 1.5U of GoTaq® (Promega). Thermocycler conditions were an initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45s, 72°C for 45 s and a final extension step of 72°C for 7 min. Specific primers were used to amplify each region containing the SNPs and restriction enzymes (NEB) chosen that would differentiate the two alleles after separation on 3% agarose gels. Two SNPs from the *CALCA* gene and two SNPs from the *RAMP1* gene were analysed as follows:
rs3781719 is the first SNP of a closely linked double base exchange at position -624 (T/C) of the *CALCA* gene promoter. The primers 5'-GCTGTTTCTCACAATATTCC and 5'-CAATTCCTGGTTGTGTGATC

generate a 109-bp PCR product which with *Bsm*AI is digested into 86- and 23-bp fragments in the case of the ‘C’ allele mutation.

rs145837941 is a SNP located in the coding sequence of the *CALCA* gene common to both calcitonin and CGRP-α. A 4218T>C base-exchange results in a non-synonymous amino acid change (Leu66Pro). The primers 5’-AGCCTGCACTGAGTTTGCTTCCC and 5’- ATCCACCTTCCTGTGTATGCTGCG generate a 236-bp PCR product which with *Alu*I is digested into 140- and 96-bp fragments in the case of the wild type ‘T’ allele.

rs3754701 is a SNP in the *RAMP1* gene promoter at position -1166 (T/A). The primers 5’-TGGCCTCTCGGCATTACTG and 5’-TGCACAGGTGGTAGGCATG generate a 373-bp PCR product which with *Acl*I is digested into 215-bp and 158-bp fragments in the case of the ‘A’ allele mutation.

rs7590387 is a SNP (G/C) located 1.4 kb downstream of the *RAMP1* gene. The primers 5’-AGAGCCTGTCGTTGTGCCCA and 5’-CTCCCGTCTCCTCGCCCTCA generate a 284-bp product which with *Xcm*I is digested into 194- and 90-bp fragments in the case of the ‘C’ allele mutation.

2.3 Statistical analysis

Hardy-Weinberg equilibrium was verified for observed genotype frequencies for each SNP to detect deviation from the normal genotype distribution in the population. Chi-square (χ^2) analysis was performed for each SNP to test for significant differences in genotype and allele frequencies in migraineurs MA, MO and combined migraine groups versus control results to detect any association with migraine. The results were also broken down into female and male sub-populations. The Statistical Package for Social Sciences (SPSS version 19.0) was used for all computations. Power analysis indicated that if the polymorphism were to confer a two-fold increase in risk of migraine, the case and control groups used in this study were of sufficient size to have ~80% power to detect an allelic association at the 0.05 level.

3. Results

In a study to investigate the role of the *CALCA* gene in psychiatric or neurological disease based on the neuromodulatory role of α-CGRP in dopaminergic transmission, Buervenich et al., (2001) identified four novel polymorphisms at the *CALCA* locus (Buervenich et al., 2001). While no positive association was found for any of these polymorphisms with Parkinson’s disease or schizophrenia, because of the important role of CGRP in migraine pathogenesis, we wished to investigate them with respect to migraine susceptibility. We previously found no association with migraine for a 16-bp deletion polymorphism in intron 1 of *CALCA* (Menon et al., 2011). However, in the present study we investigated a SNP at position -624 (T/C) of the *CALCA* gene promoter (rs3781719), which may influence transcription of α-CGRP, and the 4218T>C base-exchange (rs145837941) in the coding sequence of the *CALCA* gene common to both calcitonin and α-CGRP, which results in a non-synonymous change (Leu66Pro) of a highly conserved amino acid and is likely to disrupt structure of the CALCA propeptide. The *RAMP1* SNPs that we tested in this study were previously identified as part of a susceptibility haplotype for cerebral infarction (Nakazato et al., 2010). We were interested in the *RAMP1* promoter SNP rs3754701 at position -1166 (T/A), as it sits within a DNaseI hypersensitive site in a number of cell lines according to the ENCODE project and is also under the binding regions for a number of transcription factors including CTCF, c-myc, Znf143, GABP, CHD2 and Rad2 according to ENCODE ChIP-seq data. We also investigated rs7590387, a C/G SNP which is 1.4 kb downstream of *RAMP1*, but in a region that we found to show some association with migraine (unpublished results) in a GWAS study of the Norfolk Island population (Cox et al., 2012).

The genotypes for the two *CALCA* gene SNPs and the two *RAMP1* gene SNPs were determined using RFLP analysis. Chi-square (χ^2) analysis demonstrated that genotypes for all four SNPs were in Hardy-Weinberg equilibrium in both control and migraine groups.

***CALCA* rs3781719**

A total of 434 individuals were successfully genotyped for the *CALCA* promoter SNP, rs3781719, including 203 controls and 228 migraine cases, of which 147 were diagnosed with MA and 81 with MO. It should be noted that although MA typically accounts for only a third of migraineurs, our migraine population has a higher proportion of MA cases compared to MO, probably as the more severe MA-sufferers are more likely to identify as migrainuers and/or volunteer. χ^2-analysis was performed to compare the genotype and allele frequencies in the migraine population to controls, as well as the subgroups of migraineurs with aura to the controls and migraineurs without aura to controls (Table 1). No significant difference in genotype frequencies were found between cases and controls (p=0.260), MA and controls (p=0.563), nor MO and controls (p=0.133). There was also no significant difference in allele frequencies between cases and controls (p=0.333), MA and controls (p=0.370), nor MO and controls (p=0.593). The genotype and allele frequencies were also compared between migraineurs and controls sub-grouped into each of the genders (Table 1). No significant differences were seen between genotype or allele frequencies for female migraineurs compared to controls (p=0.270 and p=0.993, respectively). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.163) or alleles (p=0.055), although the latter is close to significance.

Table 1. Genotype and allele frequency distributions for the *CALCA* rs3781719 SNP in migraine and control groups and subgrouped by gender.

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		TT (%)	TC (%)	CC (%)			T (%)	C (%)	
Migraine	228	109 (47.8)	102 (44.7)	17 (7.5)	0.260	456	320 (70.2)	136 (29.8)	0.333
MA	147	72 (49.0)	62 (42.2)	13 (8.8)	0.563	294	206 (70.0)	88 (30.0)	0.370
MO	81	37 (45.7)	40 (49.4)	4 (4.9)	0.133	162	114 (70.4)	48 (29.6)	0.593
Control	203	111 (54.7)	75 (36.9)	17 (8.4)	-	406	297 (73.2)	109 (26.8)	-
Females									
Migraine	172	82 (47.7)	79 (45.9)	11 (6.4)	0.270	344	243 (70.6)	101 (29.4)	0.993
Control	148	76 (51.4)	57 (38.5)	15 (10.1)	-	296	209 (70.6)	87 (29.4)	-
Males									
Migraine	56	27	23	6	0.163	112	77	35	0.055

		(48.2)	(41.1)	(10.7)			(68.8)	(31.2)	
Control	55	35 (63.6)	18 (32.7)	2 (3.6)	-	110	88 (80.0)	22 (20.0)	-

MA- Migraine with aura, MO- Migraine without aura

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

N is total number of genotypes or alleles.

***CALCA* rs145837941**

A total of 462 individuals were genotyped for rs145837941, which included 236 migraine cases and 226 controls (Table 2). Because the CC genotype was very rare, the TC and CC genotypes were pooled together for analysis versus the wild type TT genotype for chi-square analysis between migraineurs and controls, however no significant difference was observed (p=0.913). Similarly there was no significant difference for alleles between cases and controls (p=0.926). No further analysis in subgroups was done because of the rarity of the C-allele. We found that rs3781719 and rs145837941 are not in linkage disequilibrium (LD) in our population (r^2=0.06, p=0.389). Furthermore, neither marker is in LD with the 16 bp deletion polymorphism that we previously genotyped in this population (r^2=0.088, p=0.205 for rs3781719 and r^2=0.042, p=0.541 for rs145837941). The HapMap data release 27 shows poor variant coverage in this region.

Table 2. Genotype and allele frequency distributions for the *CALCA* rs145837941 SNP in migraine and control groups

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		TT (%)	TC (%)	CC (%)			T (%)	C (%)	
Migraine	236	224 (94.9)	11 (4.7)	1 (0.4)	0.913	472	459 (97.2)	13 (2.8)	0.926
Control	226	214 (94.7)	12 (5.3)	0 (0)	-	452	440 (97.3)	12 (2.7)	-

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

Because of the rarity of the C-allele, the P-value for genotypes was calculated for the TT genotype versus TC and CC genotypes pooled together.

***RAMP1* rs3754701**

A total of 485 individuals were successfully genotyped for rs3754701 comprised of 243 migraine cases (broken down into 145 MA and 98 MO patients), and 242 controls (Table 3). No significant difference was found for genotype frequencies between cases and controls (p=0.370), MA and controls (p=0.276), nor MO and controls (p=0.260) and for allelic frequencies between cases and controls (p=0.376), MA and controls (p=0.912), nor MO and controls (p=0.129). The genotype and allele frequencies were also compared between migraineurs and controls of the two genders (Table 3). No significant differences were seen between genotypic or allelic frequencies for female migraineurs compared to controls (p=0.560 and p=0.819, respectively). For the male sub-population, although close, the difference between migraineurs and controls was not significant for genotypes (p=0.068), but a significant difference in the allelic frequency was observed between migraineurs and controls (p=0.031). However, to take into account the implications of multiple testing considering the entire group and sub-group analyses, Bonferroni correction the p-value to an alpha of 0.0056 renders the result non-significant.

Table 3. Genotype and allele frequency distributions for the *RAMP1* rs3754701 SNP in migraine and control groups and subgrouped by gender.

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		TT (%)	TA (%)	AA (%)			T (%)	A (%)	
Migraine	243	91 (37.4)	120 (49.4)	32 (13.2)	0.360	494	302 (61.1)	184 (38.9)	0.376
MA	145	61 (42.1)	65 (44.8)	19 (13.1)	0.276	290	187 (64.5)	103 (35.5)	0.912
MO	98	30 (30.6)	55 (56.1)	13 (13.3)	0.260	196	115 (58.7)	81 (41.3)	0.129
Control	242	94 (38.8)	126 (52.1)	22 (9.1)	-	484	314 (64.9)	170 (35.1)	
Females									
Migraine	183	71 (38.8)	89 (48.6)	23 (12.6)	0.560	366	231 (63.1)	135 (36.9)	0.819
Control	179	63 (35.2)	97 (54.2)	19 (10.6)	-	358	223 (62.3)	135 (37.7)	-
Males									
Migraine	60	20 (33.3)	31 (51.7)	9 (15.0)	0.068	120	71 (59.2)	49 (40.8)	0.031
Control	63	31 (49.2)	29 (46.0)	3 (4.8)	-	126	91 (72.2)	35 (27.8)	-

MA- Migraine with aura, MO- Migraine without aura
P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.
N is total number of genotypes or alleles.

***RAMP1* rs7590387**

A total of 456 individuals were successfully genotyped for rs7590387, consisting of 226 migraine cases (138 MA and 88 MO patients), and 230 controls. A chi-square analysis (χ^2) was performed to compare the allele and genotype frequencies of the migraine population to the controls (Table 4). No significant differences in genotypic frequencies were found between migraineurs and controls (p=0.341), MA and controls (p=0.566), nor MO and controls (p=0.299) and for allelic frequencies between migraineurs and controls (p=0.461), MA and controls (p=0.864), nor MO and controls (p=0.236). When migraineurs and controls were split into the two genders for analysis (Table 4), there were no significant differences between genotypic or allelic frequencies for female migraineurs compared to controls (p=0.865 and p=0.769, respectively), or for male migraineurs compared to controls (p=0.188 and p=0.366, respectively). According to CEPH Caucasian HapMap LD data rs3754701 and rs7590387 are not correlated (r^2=0.005) and we also find in our population that these two SNPs are not in LD (r^2=0.094, p=0.126).

Table 4. Genotype and allele frequency distributions for the *RAMP1* rs7590387 SNP in migraine and control groups and subgrouped by gender.

Group	N genotypes	Genotypes			P	N alleles	Alleles		P
		GG (%)	GC (%)	CC (%)			G (%)	C (%)	

	N				P	N			P
Migraine	226	58 (25.7)	123 (54.4)	45 (19.9)	0.341	452	239 (52.1)	213 (47.1)	0.461
MA	138	33 (23.9)	75 (54.3)	30 (21.7)	0.566	276	141 (51.1)	135 (48.9)	0.864
MO	88	25 (28.4)	48 (54.5)	15 (17.0)	0.299	176	98 (55.7)	78 (44.3)	0.236
Control	230	60 (26.1)	112 (48.7)	58 (25.2)	-	460	232 (50.4)	228 (49.6)	-
Females									
Migraine	171	44 (25.7)	93 (54.4)	34 (19.9)	0.865	342	181 (52.9)	161 (47.1)	0.769
Control	172	43 (25.7)	87 (52.1)	37 (22.2)	-	334	173 (51.8)	161 (48.2)	-
Males									
Migraine	55	14 (25.5)	30 (54.5)	11 (20.0)	0.188	110	58 (52.7)	52 (47.3)	0.366
Control	63	17 (27.0)	25 (39.7)	21 (33.3)	-	126	59 (46.8)	67 (53.2)	-

MA- Migraine with aura, MO- Migraine without aura

P-values were calculated by χ^2-analysis, significance was taken at P≤0.05.

N is total number of genotypes or alleles.

4. Discussion

The identification of susceptibility genes for complex traits such as migraine can be challenging, in particular due to the contribution of multiple, and potentially interacting, loci as well as the confounding influence of environmental factors. However, unravelling the genetic basis of migraine should improve our understanding of the pathogenesis of the disease and may suggest pathways to which therapies can be targeted. Both the *CALCA* and *RAMP1* genes are good candidates to investigate whether polymorphisms at these loci play a role in migraine pathogenesis and/or susceptibility due to the well-documented relationship of CGRP with migraine. However, neither the 16-bp deletion in intron 1 of the *CALCA* gene that we tested previously (Menon et al., 2011), nor the two *CALCA* and two *RAMP1* SNPs that we tested in this study, show a significant difference between the migraine and control groups, the MA and control groups, or the MO and control groups in this Australian population. In line with this, no positive association of the four SNPs in this study was found when the analysis was performed with female samples. An increase in frequency of the minor C-allele of the rs3781719 SNP in the promoter of the *CALCA* gene in male migraineurs was close to significance (p=0.055) and for one of the SNPs, rs3754701 which is in the promoter of the *RAMP1* gene, we observed a nominally significant increase in the frequency of the minor A-allele in male migraineurs (p= 0.031). However, the result is not significant with Bonferroni correction for multiple testing which gives a corrected p-value threshold of 0.0056. Because fewer males are affected by migraine than females, the number of male subjects in our population is low, so the association may be a false positive. However, sex-specific differences in mRNA levels of CGRP and CGRP receptor components including *RAMP1* have been found before and after induction of migraine-like symptoms in a rat preclinical migraine model (Stucky et al., 2011); furthermore, sex- specific differences are also apparent in the migraine-relevant behaviour of light aversion in mice overexpressing transgenic RAMP1 (Recober et al., 2009), suggesting that the result is potentially interesting. Future studies could look at these markers using a larger cohort, in particular of male subjects, in order to follow up any potential associations. While female sex hormones have a role in migraine and may help explain the preponderance of female migraine sufferers (Shyti et al., 2011), polymorphisms in some genes have been more strongly linked in males to either migraine, such as *DBH* (Fernandez et al., 2006) or particular migraine phenotypes, such as the C667T polymorphism in *MTHFR* (Liu et al., 2010).

The products of the *CALCA* gene including calcitonin, α-CGRP, their propeptides and other cleavage products exert many functions in the body. Mutations in a calcitonin receptor have been associated with altered bone density and decreased fracture risk in postmenopausal women (Masi et al., 1998; Taboulet et al., 1998). CGRP is vasodilator in peripheral organs as well as the brain and polymorphisms in *CALCA* gene have been linked to essential hypertension in Japanese and Chinese populations (Morita et al., 2007; Luo et al., 2008). A previous study had found no difference between migraine cases and controls for another *CALCA* gene promoter SNP (rs1553005) in a European population (Lemos et al., 2010). According to CEPH Caucasian HapMap LD data rs1553005 is in complete LD with rs3781719, however, we decided to test rs3781719 in our migraine population for a number of reasons. Firstly, we have a greater proportion of MA compared to MO cases, which would give us greater power to detect any association that is specific for this subgroup of migraine, and furthermore, our population is primarily made up of Northern European, i.e. British descendants, compared to the Southern European Portuguese cohort of that study and there is some genetic disparity between European populations. However, we found no significant association of either of the *CALCA* SNPs in this study with overall migraine or either MA or MO. The Lemos et al. (2010) study also investigated SNPs at the BDNF locus and, while no individual associations were detected, an increased risk for one of the genotype pairs of their *CALCA* SNP rs1553005 and a *BDNF* SNP in migraineurs was found, suggesting interaction between these genes in migraine susceptibility. BDNF is co-expressed with CGRP in trigeminal ganglion neurons in rats and CGRP enhances the release of BDNF (Buldyrev et al., 2006) so future studies of CGRP-related genes in migraine could focus on interactions between loci.

Many of the anti-migraine drugs currently available target some action of CGRP action. Triptans act in part by inhibiting trigeminal release of CGRP (Bigal et al., 2009), while olcegepant (BIBN-4096BS) and telcagepant (MK-0974) are CGRP receptor antagonists (Olesen et al., 2004; Ho et al., 2008; Edvinsson and Ho, 2010). Some migraineurs respond better with certain migraine drugs than others and some fail to respond at all; studies have investigated SNPs in some factors and genes that may be involved (Ishii et al., 2012). Although we failed to detect association with migraine for the *CALCA* and *RAMP1* variations that we have looked at in this study, these genes could also potentially play a role in drug response to anti-migraine agents.

In conclusion, we investigated whether the SNPs rs3754701 and rs7590387 in *RAMP1* and rs3781719 and rs145837941 in the *CALCA* locus are associated with migraine susceptibility. We find no significant association of any of the SNPs tested with migraine overall. We did detect a nominally significant association of the minor A-allele for *RAMP1* rs3754701 in male migraine subjects, but this was no longer significant after Bonferroni correction. Although RAMP1, via its interaction with CGRP, has been implicated in migraine (Recober et al., 2009), our study is the first to investigate polymorphisms in the *RAMP1* gene in a migraine population. Future studies using a larger population to increase the power to detect significant associations, and greater gene coverage and haplotype analysis, would clarify whether polymorphisms in CGRP-related genes play a role in migraine.

Author's contributions

H.G.S., J.B. and S.M. performed the experimental procedures, participant sample preparation and contributed towards statistical analysis and manuscript finalisation. R.A.L. and L.M.H. contributed towards data interpretation and finalisation of the manuscript. L.R.G. and E.A.M. participated in the conception and design of the study. L.R.G. was also involved in data analysis and interpretation and coordinated the study.

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